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Synthesis and Biological Characterization of Amidopropenyl Hydroxamates as HDAC Inhibitors

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A series of amidopropenyl hydroxamic acid derivatives were prepared as novel inhibitors of human histone deacetylases (HDACs). Several compounds showed potency at <100 nM in the HDAC inhibition assays, sub-micromolar IC_{50} values in tests against three tumor cell lines, and remarkable stability in human and mouse microsomes was observed. Three representative compounds were selected for further characterization and submitted to a selectivity profile against a series of class I and class II HDACs as well as to preliminary in vivo pharmacokinetic (PK) experiments. Despite their high microsomal stability, the compounds showed medium-to-high clearance rates in in vivo PK studies as well as in rat and human hepatocytes, indicating that a major metabolic pathway is catalyzed by non-microsomal enzymes.

Introduction

Reversible post-translational histone modifications such as acetylation and deacetylation play an essential role in modifying chromatin structure and regulating gene transcription in eukaryotic cells. The acetylation status is tightly controlled by counteracting enzyme families: histone acetyltransferases (HATs), enzymes that catalyze histone acetylation, which is generally associated with transcriptional activation, and histone deacetylases (HDACs), enzymes responsible for deacetylation of lysine residues located at the N termini of the proteins; this is normally correlated with transcriptional repression.[1,2] HDACs can be grouped into two distinct families: Zn^{2+}-dependent enzymes, also known as "classical HDACs", and the NAD^{+}-dependent sirtuins. Based on phylogenetic conservation, histone deacetylases can be further grouped into four classes: class I (HDACs 1–3 and 8), II (HDACs 4–7, 9, and 10), and IV (HDAC 11) belong to the classical HDACs, whereas sirtuins are represented by class III enzymes.[3,4] Aberrant regulation of histone acetylation as well as acetylation of non-histone proteins has been shown to be linked not only to the pathogenesis of cancer, but also to several other diseases.[5–12] Over the past few years, there has been a growing interest in the development of HDAC inhibitors, and a common pharmacophore based on the first compounds has been presented by Miller et al.[13] This model pharmacophore is composed of a metal binding group, such as a hydroxamic acid, a linking group consisting of linear or cyclic structures, and a surface recognition domain, which is generally a hydrophobic group. Many studies have demonstrated that HDAC inhibition leads to antitumor effects, and several compounds are currently in clinical studies for solid and hematological tumor therapy, Huntington's disease, amyotrophic lateral sclerosis, and fungal infections.[14–16] Currently, two HDAC inhibitors have gained approval by the US Food and Drug Administration (FDA) for the treatment of cutaneous T-cell lymphoma: vorinostat (**1**; Zolinza, also known as SAHA or suberoylanilide hydroxamic acid),[17–19] which was approved in October 2006, and the cyclic depsipeptide rhomidepsin (**2**; FK228, Gloucester),[20] which was approved three years later in November 2009. Further compounds in clinical studies include hydroxamic acids such as panobinostat (**3**; LBH589, Novartis, phase III),[21] belinostat (**4**; PXD101, Topotarget, phase III),[22] and givinostat (**5**; ITF-2357, Italfarmaco, phase II),[23] as well benzamides, such as entinostat (**6**; SNDX-275, Syndax, phase II)[24] and mocetinostat (**7**; MGCD-0103, Methylgene, phase II).[25] A further class comprises short fatty acids, such as sodium phenylbutyrate (**8**; Tikvah Therapeutics, phase II).[26,27]

We recently described a novel HDAC inhibitor scaffold, namely aryloxypropenylphenyl- and aryloxypropenylpyridinyl

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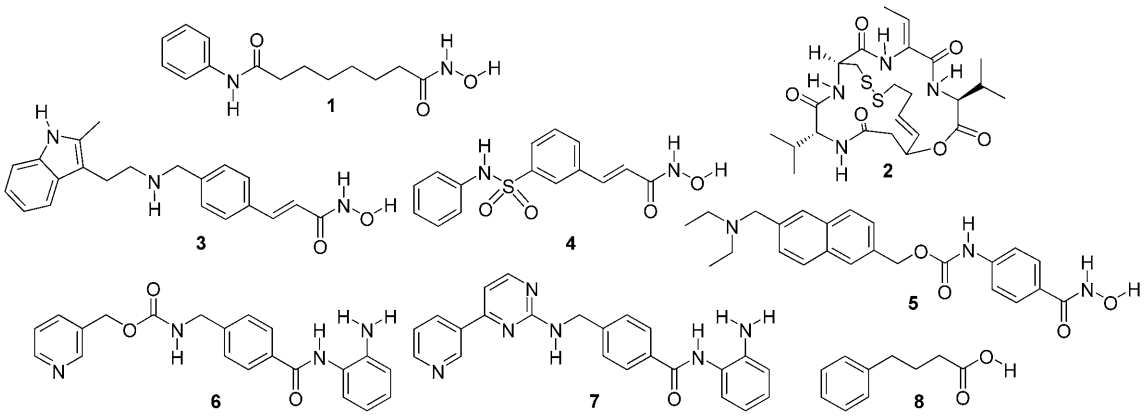
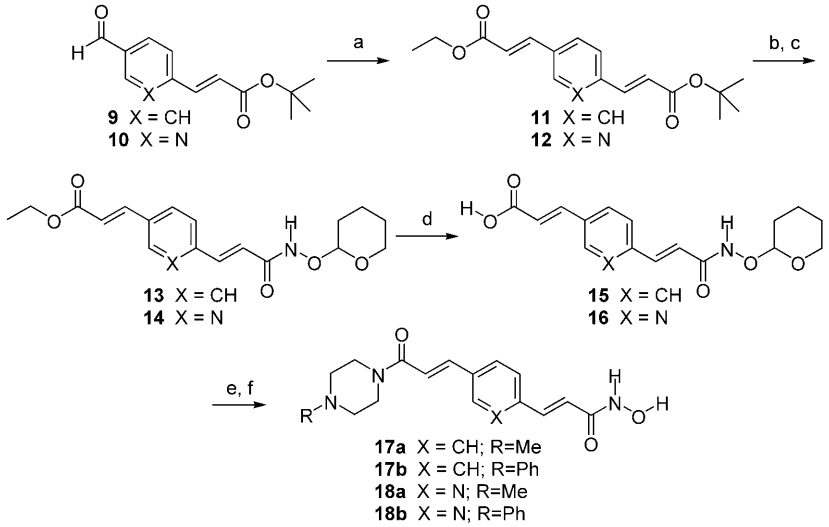


hydroxamates.[28] The study was designed to obtain potent inhibitors with improved pharmacokinetic (PK) properties. As outlined in the work, unsubstituted aryloxypropenyl derivatives have poor solubility and were rapidly metabolized in mouse microsomes, whereas the introduction of a solubilizing 4-methylpiperazin-1-yl moiety led to more soluble structures with enhanced microsomal stability and a better PK profile. In a further experiment, metabolic profiling of (*E*)-*N*-hydroxy-3-[4-((*E*)-3-oxo-3-phenyl-1-propen-1-yl)phenyl]acrylamide performed in rat hepatocytes showed that reduction of the α,β-unsaturated ketone was one of the major route of metabolism for the molecule.[29]



Scheme 1. Reagents and conditions: a) THF, NaH, triethyl phosphonoacetate, RT; b) CH_2Cl_2, TFA, RT; c) CH_2Cl_2, EDC, HOBt, TEA, NH_2OTHP, RT; d) 1 M LiOH, THF, RT; e) DMF, EDC, HOBt, TEA, 4-R-piperazine; f) CH_2Cl_2, Et_2O, HCl, RT.

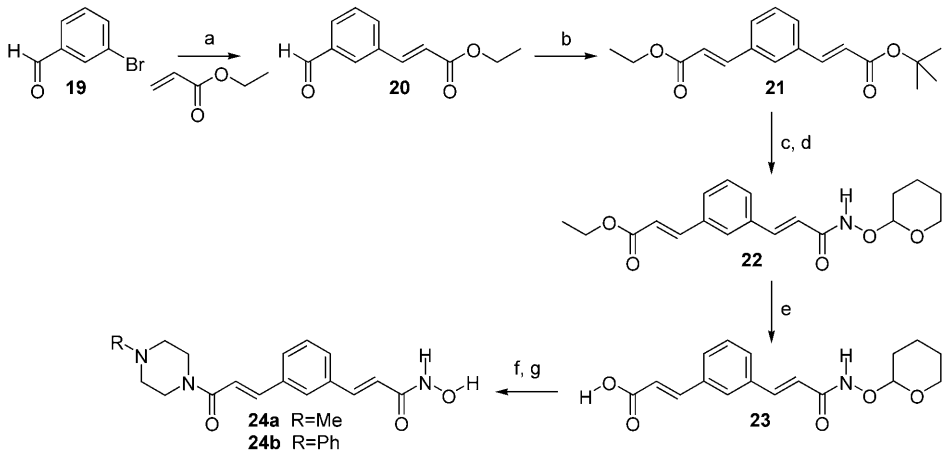
Herein we describe the synthesis and biological activity of amidopropenyl hydroxamic acid derivatives, which are structurally related to the series described above and are devoid of the potentially metabolically unstable α,β-unsaturated ketone moiety.

Results and Discussion

Chemistry

The syntheses of the hydroxamic acid derivatives **17a**,**b** and **18a**,**b** were carried out by starting from (*E*)-*tert*-butyl-3-(4-formylphenyl)acrylate (**9**)[30] and *tert*-butyl-(*E*)-3-(5-formylpyridin-2-yl)acrylate (**10**).[28] As illustrated in Scheme 1, the *tert*-butylacrylates **9** and **10** were treated with triethyl phosphonoacetate according to Horner–Emmons conditions, giving the diesters **11** and **12**. Thus, the *tert*-butyl group was cleaved with trifluoroacetic acid (TFA) in dichloromethane, and the obtained acrylic acid was then treated with *O*-(2-tetrahydropyranyl)hydroxylamine (NH_2OTHP) in the presence of *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxybenzotriazole (HOBt) as coupling agents, leading to the tetrahydropyran-2-yl-protected hydroxamic acids **13** and **14**. Hydrolysis of the ethyl ester with lithium hydroxide in tetrahydrofuran, amide construction via standard amide coupling, and final deprotection of the tetrahydropyranyl moiety with hydrochloric acid in diethyl ether provided the desired hydroxamic acids **17a**,**b** and **18a**,**b**.

Heck reaction of the commercially available bromo derivative **19** with ethyl acrylate in the presence of palladium acetate, triphenylphosphine, triethylamine, and sodium bicarbonate furnished (*E*)-3-(3-formylphenyl)acrylic acid ethyl ester (**20**, see Scheme 2), which was then converted into the diester **21** under Horner–Emmons conditions using *tert*-butyldiethyl phosphonoacetate. Subsequent removal of the *tert*-butyl ester group with TFA, coupling of the carboxylic acid with NH_2OTHP using the water-soluble carbodiimide reagent EDC and HOBt gave the tetrahydropyran-2-yl-protected hydroxamic acid **22**. Deprotection of the ethyl ester, EDC coupling of the resulting acid with the corresponding cyclic amines, and final deprotection of the THP group gave the desired amidopropenyl hydroxamic acid derivatives **24a**,**b**.



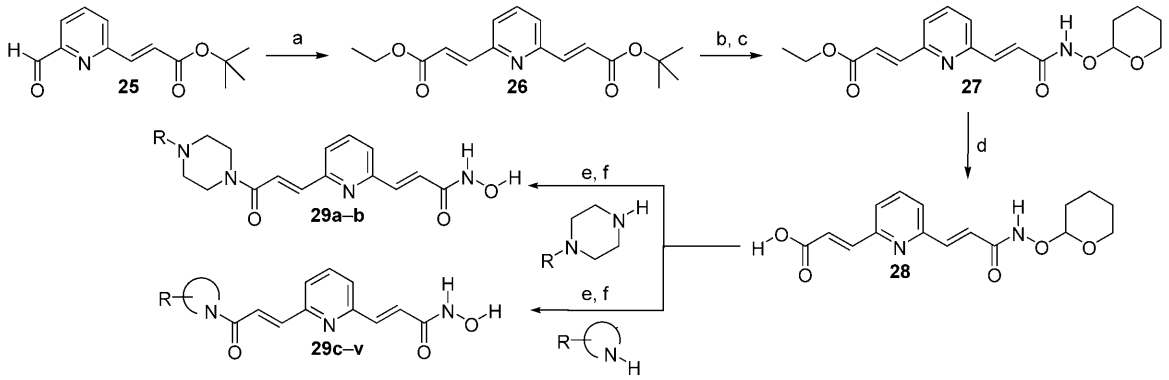
Scheme 2. Reagents and conditions: a) DMF, TEA, $NaHCO_3$, PPh_3, $Pd(OAc)_2$, 100 °C; b) NaH, THF, *tert*-butyldiethyl phosphonoacetate, 0 °C, then RT; c) CH_2Cl_2, TFA, RT; d) CH_2Cl_2, EDC, HOBt, TEA, NH_2OTHP, RT; e) 1 M LiOH, THF, RT; f) CH_2Cl_2, DMF, EDC, HOBt, TEA, 4-R-piperazine, RT; g) CH_2Cl_2, Et_2O, HCl, RT.

The diester **26** was prepared by Horner–Emmons reaction of (*E*)-3-(6-formylpyridin-2-yl)acrylic acid *tert*-butyl ester (**25**)[28] and triethyl phosphonoacetate (Scheme 3). Deprotection of the *tert*-butyl group and reaction with NH_2OTHP gave the tetrahydropyranyl-protected hydroxamic acid **27**. Subsequent cleavage of ethyl ester in 4 N sodium hydroxide in THF led to the pyridine acrylic acid **28**. Amide formation and deprotection of the hydroxamic acid following the conditions described in Schemes 1 and 2 afforded the desired *N*-hydroxyacrylamides **29a**–**v**.

Biology

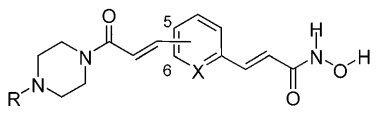
The compounds were profiled using HeLa cell nuclear extracts as a source of class I, II, and IV HDAC enzymatic activity. The absence of NAD^+ under the experimental conditions allowed exclusion of the contribution of class III sirtuins to the enzymatic activity. Subsequently, their antiproliferative potency was assessed using the chronic myelogenous leukemia cell line K562, human alveolar basal epithelial lung cancer A549 cells, and human colon cancer HCT116 cells. Initially, a series of piperazine oxypropenyl analogues with various linkers were tested. For this purpose, 4-methyl- and 4-phenylpiperazinyl analogues of the following four linkers were prepared: *meta*- and *para*-substituted phenylacrylate, and pyridin-2-ylacrylate substituted at positions 5 or 6. As summarized in Table 1, the *meta*-substituted phenylacrylate derivatives **24a** and **24b** exhibited IC_{50} values of 0.16 and 0.35 μM, respectively, and were about 10-fold more potent than the corresponding *para*-substituted compounds **17a** and **17b**. In the pyridinyl series, the differences between 2,5- and 2,6-disubstituted pyridin-2-ylacrylates was less evident: **29b** was about fourfold more potent than **18b**, whereas the two *N*-methylpiperazine derivatives **29a** and **18a** were almost equipotent. Comparison of the compounds containing a central phenyl ring with those with a pyridinyl ring showed that the latter inhibitors were generally more potent in the HDAC assay. Similar observations had also been found for the previously described aryloxypropenylphenyl- and aryloxypropenylpyridinyl hydroxamates.[28]

Antiproliferative activities were overall superior for those compounds containing the phenylpiperazine group; the methylpiperazine analogues generally exhibited IC_{50} values >10 μM in the cellular assays. Limited permeability for these polar hydroxamic acids could explain the absence of antiproliferative activity observed at the doses studied. Although **29b** was ~5–6-fold more potent than **18b** and **24b** in the HDAC assay, the three compounds exhibited similar antiproliferative activities in K562 and HCT116 cells. Slightly higher IC_{50} values in the A549 cell line were observed for the three compounds. Linking the activity observed in a biochemical assay with cellular potency on the basis of the physicochemical properties of a molecule is often difficult. Several variables are known to play a role, such as cellular membrane permeability, subcellular compartmentalization, and cellular mechanisms for efficiently extruding xeno-



Scheme 3. Reagents and conditions: a) NaH, THF, triethyl phosphonoacetate, RT; b) CH_2Cl_2, TFA, RT; c) CH_2Cl_2, EDC, HOBt, TEA, NH_2OTHP, RT; d) 4 M NaOH, THF, RT; e) CH_2Cl_2, DMF, EDC, HOBt, TEA; f) CH_2Cl_2, Et_2O, HCl, RT.

Table 1. SAR of 4-methyl- and 4-phenylpiperazinyl analogues **17a**,**b**, **18a**,**b**, **24a**,**b**, and **29a**,**b**.[a]



Compd	X	Position	R	IC_{50} [μM] HDAC	K562	A549	HCT116	Microsomes [%] mouse	human	Solubility [μM]
17a	CH	5	CH_3	1.25	14.5	26.8	7.45	58	56	384
17b	CH	5	Ph	5.60	3.37	7.32	5.77	27	51	5
18a	N	5	CH_3	0.438	8.84	22.9	4.37	88	87	430
18b	N	5	Ph	0.299	1.41	3.68	1.33	91	82	10
24a	CH	6	CH_3	0.164	19.1	32.1	12.6	82	100	477
24b	CH	6	Ph	0.354	0.980	1.38	0.780	72	60	76
29a	N	6	CH_3	0.345	21.7	>50	16.0	72	65	434
29b	N	6	Ph	0.069	1.13	2.43	1.07	74	77	214

[a] Assays were performed in replicate ($n \geq 2$); values are shown are the mean, and standard deviations are $\leq 25\%$ of the mean.

biotics. Compound **17b** exhibited, in line with the limited enzymatic inhibition, a lower antiproliferative activity in all three cell lines relative to the three inhibitors **18b**, **24b**, and **29b**. The inhibitors were then submitted to a microsomal stability assay, and their solubility was assessed at pH 7.4. Compounds were exposed to mouse and human microsomes at 37 °C for 30 min, and the percentage of the unmetabolized derivative was determined by LC–MS–MS. With the exception of **17b**, all compounds were metabolized by $<50\%$ under the test conditions used both in mouse and human microsomes. In comparison, previously disclosed unsubstituted aryloxypropenyl derivatives[28] were shown to be rapidly metabolized in mouse and human microsomes. The compounds described herein (which contain an amidic moiety instead of the ketone group) demonstrated significantly higher stability in human and mouse microsome preparations. This is in agreement with the identification of the α,β-unsaturated ketone moiety as a major metabolic pathway of the previously described *N*-hydroxy-3-[4-((*E*)-3-oxo-3-phenyl-1-propen-1-yl)phenyl]acrylamide.[29]

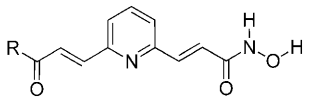
All four 4-methylpiperazinyl analogues **17a**, **18a**, **24a**, and **29a** exhibited good aqueous solubility at neutral pH. Major differences were found among the 4-phenylpiperazines: the two *para*-substituted phenyl- and pyridin-2-ylacrylates **17b** and **18b** had a much lower aqueous solubility (5 and 10 μM, respectively) than the corresponding *meta* analogues **24b** and **29b** (76 and 214 μM, respectively). The hydroxamic acid **29b** was selected as a starting point for further exploration based on the biochemical and cellular potency and the in vitro ADME properties. The inhibitor was the most potent compound within this group in the enzymatic assay ($IC_{50} = 69$ nM); furthermore, it was found to be more soluble than **24b**. Because hydrophobic aromatic groups represent a common surface recognition domain in several HDAC inhibitors,[13] a series of cyclic amine derivatives substituted by phenyl were synthesized. The results of the biological tests are reported in Table 2. HDAC IC_{50} values varied from 0.025 μM (compound **29e**) to 0.294 μM (compound **29c**). The limited differences in activities (except for the outlier **29e**) can be ascribed to the fact that the surface region of the HDAC enzyme is highly flexible and is able to accommodate inhibitors with various capping groups.[31,32] Antiproliferative activities in A549 cells ranged between 0.61 μM (**29e**) and 6 μM (**29g**), whereas differences in cellular activities of these inhibitors were minor in the other two cell lines. Compound **29e** ($IC_{50} = 0.44$ μM) was about fourfold more potent than **29f** in K562 cells and about fivefold more active than **29g** in HCT116 cells ($IC_{50} = 0.29$ μM).

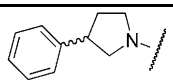
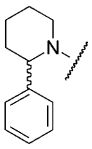
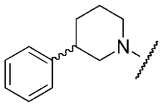
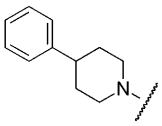
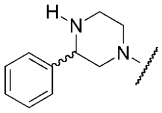
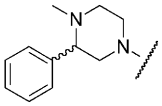
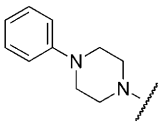
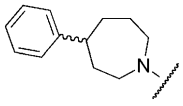
All compounds (with the exception of **29i** with 29% remaining in mouse microsomes) exhibited good stability in mouse and human microsomes, with $<50\%$ of the inhibitors metabolized after 30 min incubation. Furthermore, aqueous solubility of all compounds exceeded 100 μM at pH 7.4.

The good potency of the compounds, together with the favorable ADME profile, prompted us to explore further modifications of the surface recognition domain. Compound **29e** was selected as a starting point for further exploration based on its good biochemical and antiproliferative activity, and a series of substituted 3-phenylpiperidine analogues were synthesized. As shown in Table 3, the *R* and *S* isomers of compound **29e** were equipotent in the enzymatic and cell-based assays. Introduction of a fluoro, methyl, or methoxy substituent at the *ortho*, *meta*, and *para* positions of the phenyl ring led to compounds that did not show substantial differences in the enzymatic assay, whereas the two derivatives **29u** and **29v**, with a naphthyl cap group in place of the phenyl ring, were 10-fold less potent than **29e**. All compounds displayed good antiproliferative activity in all three cell lines tested, particularly K562 cells, with IC_{50} values <1 μM. Compound **29l**, the 2-fluorophenyl analogue of **29e**, emerged as the most potent agent, with an IC_{50} value of 0.068 μM. Compound **29l** was also found to be the most active compound in the other two cell lines, with IC_{50} values of 0.057 μM (HCT116) and 0.196 μM (A549).

This sub-series also showed good microsomal stability in general. A remarkable decrease in stability was observed between the 4-methylphenyl and 4-methoxyphenyl analogues **29q** and **29t**, respectively, and the unsubstituted compound **29e** in human microsomes. Specifically, the methyl derivative **29q** was almost completely metabolized after 30 min, and just 25% of the methoxy analogue **29q** remained unaltered, whereas $<20\%$ of **29e** was degraded under the experimental conditions. This trend, however, was not observed in mouse

Table 2. SAR of phenylcycloamine analogues **29b–i**.[a]



Compd	R	IC_{50} [μM] HDAC	K562	A549	HCT116	Microsomes [%] mouse	human	Solubility [μM]
29c		0.294	1.220	4.670	1.300	66	66	307
29d		0.269	0.616	1.230	0.432	52	90	218
29e		0.025	0.445	0.610	0.294	74	86	286
29f		0.146	1.730	2.788	1.470	75	98	167
29g		0.100	1.550	6.350	1.660	82	88	345
29h		0.173	0.464	1.510	0.390	78	85	365
29b		0.069	1.126	2.430	1.070	74	77	214
29i		0.286	0.568	1.890	0.581	29	46	117

[a] Assays were performed in replicate ($n \geq 2$); values are shown are the mean, and standard deviations are $< 30\%$ of the mean.

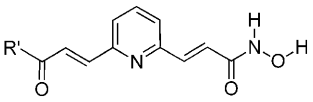
microsomes. On the other hand, the 4-fluorophenyl derivative **29n** was fully recovered after 30 min incubation in mouse and human microsome preparations. Table 3 also includes the solubility data for this sub-series; the two naphthyl analogues **29u** and **29v** were virtually insoluble at pH 7.4, whereas the aqueous solubility of **29l** and **29m** exceeded 400 μM.

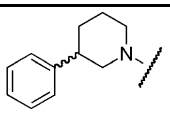
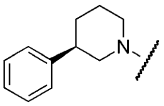
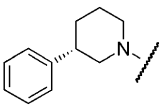
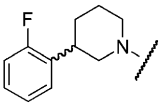
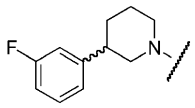
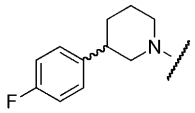
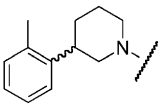
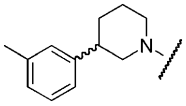
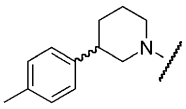
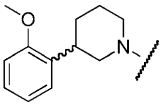
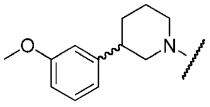
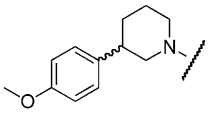
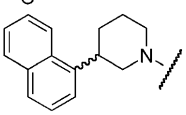
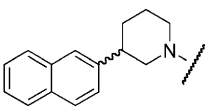
The arylhydroxamate **24b** and the two heteroaryl hydroxamates **29b** and **29e**, which had been chosen as starting points for further explorations, were selected for a more extensive characterization. In an initial experiment the selectivity profile against HDAC subtypes 1, 3, 4, 6, and 8 was evaluated. The results are summarized in Table 4 together with the data obtained for SAHA (**1**), which are in agreement with recently published data.[33] The potency of the three tested compounds as well of **1** was in the nanomolar range against the class I and II HDACs, with the exception of HDAC8, for which the IC_{50} values were ~1 μM. The three structures can be classified as typical pan-HDAC inhibitors upon comparison of the biological profile with the data of **1** and other previously reported hydroxamic acid derivatives.[5,34]

A further experiment was designed to study changes in basal acetylation levels in K562 leukemia cells in the presence of the inhibitors at a final concentration of 0.5 μM. The results are included in Table 4; consistent with the ability to inhibit cellular HDACs, all compounds were able to induce an increase in basal acetylation levels greater than threefold relative to untreated K562 cells.

The three selected compounds **29b**, **29e**, and **24b** were then submitted to PK studies in mice. The compounds were administrated in single intravenous (i.v.) or oral doses of 5 or 15 mg kg^{-1}, respectively. The derivatives were dissolved in water containing 3% DMSO and 10% hydroxypropyl-β-cyclodextrin (Encapsin™) for the i.v. dose, or in water (**29b**) or water containing 5% DMSO and 9.5% Encapsin (**24b**, **29e**) for the oral dose. The main plasma PK parameters are presented in Table 5. For comparison, Table 5 also lists previously published PK results for SAHA (**1**) obtained in house.[35] Following i.v. ad-

Table 3. SAR of 3-phenylpiperidine analogues **29e** and **29j–v**.[a]



Compd	R	IC_{50} [μM] HDAC	K562	A549	HCT116	Microsomes [%] mouse	human	Solubility [μM]
29e		0.025	0.445	0.610	0.294	74	86	286
29j		0.025	0.214	0.570	0.196	57	77	174
29k		0.031	0.229	0.533	0.169	70	75	187
29l		0.020	0.068	0.196	0.057	48	66	424
29m		0.033	0.249	0.757	0.240	59	69	427
29n		0.026	0.462	1.050	0.291	100	100	113
29o		0.020	0.309	0.598	1.990	54	89	102
29p		0.097	0.519	0.898	0.516	59	30	93
29q		0.092	0.655	1.127	0.405	62	4	52
29r		0.089	0.640	1.780	0.796	39	61	90
29s		0.021	0.186	0.688	0.242	58	20	142
29t		0.068	0.299	0.948	0.383	70	25	125
29u		0.307	0.764	1.560	0.601	37	52	15
29v		0.215	0.390	0.763	0.284	92	100	1

[a] Assays were performed in replicate ($n \geq 2$); values are shown are the mean, and standard deviations are $\leq 30\%$ of the mean.

Table 4. HDAC selectivity profile and histone acetylation assay for **1**, **24b**, **29b**, and **29e**.[a]

Compd	IC_{50} [μM]					AI[b]
	HDAC1	HDAC3	HDAC8	HDAC4	HDAC6	
1	0.0453	0.0149	1.65	0.0377	0.0115	3.61
24b	0.203	0.028	1.88	0.303	0.038	5.0
29b	0.098	0.030	1.56	0.150	0.025	4.5
29e	0.025	0.005	0.86	0.024	0.016	4.2

[a] Assays were performed in replicate ($n \geq 2$); values are shown are the mean, and standard deviations are $< 30\%$ of the mean. [b] Acetylation increment at an inhibitor concentration of 0.5 μM.

Table 5. Pharmacokinetic profiles for **1**, **24b**, **29b**, and **29e**.

Parameter[a]	Compd			
	1	**24b**	**29b**	**29e**
$AUC_{i.v.\,0-\infty}$ [ng h mL^{-1}]	657	440	590	1191
$t_{1/2}$ [h]	0.5	1.22	0.27	0.67
CL [L h^{-1} kg^{-1}]	7.62	11.37	8.47	4.19
V_{ss} [L kg^{-1}]	1.2	5.2	0.8	0.55
F [%]	12.1	10	2.9	3.1

[a] AUC: area under the plasma concentration–time curve of the drug; $t_{1/2}$: half-life; CL: volume of plasma cleared of the drug per unit time; V_{ss}: volume of distribution at the steady state defined by the amount of drug in the body over the concentration of the drug in the plasma at the steady state; F: percentage of the dose reaching blood circulation after oral administration.

ministration, the elimination half-life was found to be between 0.27 h (**29b**) and 1.22 h (**24b**). Furthermore, the three compounds as well as the reference compound **1** exhibited high clearance rates. In particular, inhibitors **24b** and **29b** (as well as reference compound **1**) showed clearance rates that exceeded the hepatic blood flow of 5.4 L h^{-1} kg^{-1} in mice.[35] The steady-state volume of distribution V_{ss} for **29b** and **29e** was in the range of the total body volume, indicating a moderate tissue distribution of these compounds. On the other hand, **24b** exhibited a high V_{ss} value of 5.2 L kg^{-1}. Similarly to reference compound **1**, the three amidopropenyl hydroxamates displayed a low oral bioavailability in mice with $F \leq 10\%$.

Overall, despite good in vitro ADME data (particularly the excellent stability of the compounds in human and mouse microsomes), the three selected derivatives showed surprisingly low oral bioavailability in the PK studies in mice. However, limited oral bioavailability and high clearance rates in rodent PK experiments were also observed for several other HDAC inhibitors containing a hydroxamic acid group.[36,14]

The differences between the in vitro and in vivo properties, particularly between the in vitro microsomal stability and the high in vivo clearance of the three representative amidopropenyl derivatives, prompted us to perform a set of in vitro studies in order to find plausible explanations. First, the three selected inhibitors were submitted to a plasma stability assay. The compounds were incubated at a concentration of 2.5 μM in mouse plasma. After incubation for 1 h at 37 °C, a 50 μL aliquot of plasma was removed, quenched with acetonitrile (140 μL), centrifuged, and the supernatant was analyzed by LC–MS–MS. All three test compounds proved to be stable under the conditions used, and the recovery of the products exceeded 85%.

Once the stability of the three compounds in plasma was confirmed, the next step was to explore their clearance behavior in greater detail. For this purpose the inhibitors were incubated in rat and human hepatocytes in order: 1) to explore the metabolism in an additional species (rat), and 2) to determine the metabolic behavior of the derivatives in hepatocytes relative to microsomes. Considering previously described clearance classifications,[37,38] the three amidopropenyl hydroxamates underwent a rapid biotransformation in rat and human hepatocytes (Table 6). The clearance rates were overall higher in rat than in human hepatocytes, particularly for **24b**. A high clearance rate was also observed for reference compound **1** in rat hepatocytes (57.1 μL min^{-1} per 10^6 cells). On the other hand, compound **1** showed a higher stability in human hepatocytes than the three amidopropenyl hydroxamic acid derivatives. These results reflected the observed in vivo PK data obtained in mice, and showed that the metabolic stability observed in microsomes was not found in hepatocytes.

Table 6. Clearance rates in rat and human hepatocytes for **1**, **24b**, **29b**, and **29e**.

Compd	CL [μL min^{-1} (10^6 cells)$^{-1}$]	
	Rat	Human
1	57.1	4.60
24b	114.0	14.5
29b	23.3	11.5
29e	46.3	21.9

A similar outcome has been described by Elaut et al.,[39] who studied the in vitro biotransformation of trichostatin A (TSA). TSA was rapidly and completely metabolized by rat hepatocytes, whereas the compound was metabolized slowly and incompletely in rat and human microsome preparations. Two major metabolites found in the hepatocytes were TSA amide, which was obtained by reduction of the hydroxamic acid group to amide, and N-monodemethylated TSA. The demethylated metabolite was also detected in the microsomal preparation, but not the TSA amide.

The rat and human hepatocyte preparations of compounds **24b**, **29b**, and **29e** were submitted, together with reference compound **1**, to LC–MS–MS analysis and indeed, amidic metabolites were detected in all samples. Thus, as further characterization, a biotransformation experiment of **29b** and **29e** was carried out. The compounds were incubated at 10 μM in 10^6 cells μL^{-1} at 37 °C. After 90 min the reaction was stopped with acetonitrile, and LC–MS–MS analysis showed that **29b** and **29e** were metabolized 77 and 86%, respectively, and that the amidic analogues were the major metabolites in both cases. A further hydroxylated metabolite of **29e** was found, while no relevant metabolites were detected for **29b**. These results are an additional confirmation that a major metabolic pathway of hydroxamic acid derivatives may be catalyzed by non-microsomal enzymes, and that biotransformation investi-

gations in microsome preparations may not be predictive of the in vivo clearance behavior of the studied examples.

Conclusions

In summary, the synthesis and biological characterization of novel amidopropenyl hydroxamic acid derivatives has been described. Several compounds showed good in vitro potency together with significant microsomal stability. Three representative compounds, **24b**, **29b**, and **29e**, demonstrated nanomolar potency against class I (HDACs 1, 3, and 8) and class II (4 and 6) HDACs, showing a profile similar to other previously described pan-HDAC inhibitors. The remarkable stability of the compounds in microsomes, however, was not confirmed by in vivo PK experiments in mice. Additional studies indicated that the three compounds showed high clearance rates in rat and human hepatocytes, suggesting that a major metabolic pathway for the hydroxamic acid derivatives may be catalyzed by non-microsomal enzymes. Reduction of the hydroxamic acid group to its corresponding amide was identified for the three compounds as well as for SAHA as one relevant biotransformation pathway. Similar observations have been previously described for the HDAC inhibitor TSA.[39]

Experimental Section

Chemistry

All reagents and solvents were of commercially available reagent grade and were used without further purification. Flash chromatography was performed on Merck silica gel 60 (0.04–0.063 mm). ^{1}H and ^{13}C NMR spectroscopic data were recorded on a Bruker spectrometer at 300 and 75 MHz, respectively, with $(CH_3)_4Si$ as internal standard, and, unless stated otherwise, at 300 K. Chemical shift (δ) values are given in ppm, and coupling constants (J) are expressed in Hz. Standard abbreviations indicating multiplicity are used as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, and bs = broad signal. HPLC–MS experiments were performed with a Waters 2777 sample manager and a Waters 1525 binary HPLC pump equipped with a Waters 2996 diode array and a Micromass ZQ 2000 single quadrupole (Waters), or on an Acquity UPLC apparatus equipped with a diode array and a Micromass ZQ single quadrupole (Waters). HPLC purity of the target compounds was assessed either with a Waters 2777 sample manager and a Waters 1525 binary HPLC pump equipped with a Waters 2996 diode array and a Micromass ZQ 2000 single quadrupole (Waters) (Methods 1 and 5), or on an Acquity UPLC apparatus equipped with a diode array and a Micromass ZQ single quadruple (Waters) (all other methods). The flow rate was adjusted to 0.7 mL min^{-1} for Method 5 and 0.6 mL min^{-1} for the other methods, and the splitting ratio between the amount submitted to the mass spectrometer and to the waste was 1:4. Mobile phase A consisted of a mixture of H_2O and CH_3CN (95:5) containing 0.1% TFA, and mobile phase B consisted of a mixture of H_2O and CH_3CN (5:95) containing 0.1% TFA. Purity refers to UV detection at λ 254 nm. High-resolution mass spectrometry experiments were performed on an Autoflex III smartbeam vertical MALDI-TOF (TOF/TOF) mass spectrometer (Bruker Daltonics).

Method 1: Atlantis dC$_{18}$ (3 μm, 2.1×100 mm) column; gradient: 0–0.50 min (A: 98%, B: 2%), 0.50–6.00 min (A: 0%, B: 100%), 6.00–7.00 min (A: 0%, B: 100%), 7.00–7.10 min (A: 98%, B: 2%); 7.10–8.50 min (A: 98%, B: 2%).

Method 2: BEH C$_{18}$ (1.7 μm, 2.1×50 mm) column; gradient: 0–0.25 min (A: 100%, B: 0%), 0.25–3.30 min (A: 0%, B: 100%), 3.30–4.00 min (A: 0%, B: 100%), 4.00–4.10 min (A: 100%, B: 0%); 4.10–5.00 min (A: 100%, B: 0%).

Method 3: BEH C$_{18}$ (1.7 μm, 2.1×50 mm) column; gradient: 0–0.25 min (A: 98%, B: 2%), 0.25–3.30 min (A: 0%, B: 100%), 3.30–4.00 min (A: 0%, B: 100%), 4.00–4.10 min (A: 98%, B: 2%); 4.10–5.00 min (A: 98%, B: 2%).

Method 4: BEH C$_{18}$ (1.7 μm, 2.1×50 mm) column; gradient: 0–0.25 min (A: 95%, B: 5%), 0.25–3.30 min (A: 0%, B: 100%), 3.30–4.00 min (A: 0%, B: 100%), 4.00–4.10 min (A: 95%, B: 5%); 4.10–5.00 min (A: 95%, B: 5%).

Method 5: Synergi (2.5 μm, 2.0×20 mm) column; gradient: 0–0.25 min (A: 95%, B: 5%), 0.25–3.5 min (A: 0%, B: 100%), 3.35–4.50 min (A: 0%, B: 100%), 4.50–4.60 min (A: 95%, B: 5%); 4.60–6.00 min (A: 95%, B: 5%).

Method 6: BEH C$_{18}$ (1.7 μm, 2.1×50 mm) column; gradient: 0–0.50 min (A: 95%, B: 5%), 0.50–6.00 min (A: 0%, B: 100%), 6.00–7.00 min (A: 0%, B: 100%), 7.00–7.10 min (A: 95%, B: 5%); 7.10–8.50 min (A: 95%, B: 5%).

(*E*)-*N*-Hydroxy-3-{4-[(*E*)-3-(4-phenylpiperazin-1-yl)-3-oxo-1-propen-1-yl]phenyl}acrylamide (17b): (*E*)-*tert*-Butyl-3-(4-formylphenyl)acrylate[30] (**9**, 850 mg, 3.66 mmol) was dissolved in dry THF (5 mL) and added dropwise to a stirred mixture of triethyl phosphonoacetate (0.878 mL, 4.43 mmol) and NaH (60% oil dispersion, 228 mg, 5.7 mmol) in dry THF (5 mL). The resulting solution was stirred at room temperature for 7 h and then additional NaH (60% oil dispersion, 100 mg, 2.5 mmol) was added. After stirring overnight at room temperature the reaction was quenched with H_2O and the mixture was extracted with Et_2O. The organic phase was dried over Na_2SO_4 and evaporated in vacuo to afford ethyl (*E*)-3-[4-((*E*)-2-*tert*-butoxycarbonylvinyl)phenyl]acrylate (**11**) (1.1 g), which was used in the next step without further purification; LC–MS (ESI): *m/z*: 303 $[M+H]^+$.

A solution of the *tert*-butyl ester **11** (2.75 g, 9.10 mmol) and TFA (7 mL) in CH_2Cl_2 (20 mL) was stirred at room temperature for 1 h. The solvent was removed in vacuo to give the acrylic acid as a white solid (2.13 g, 95%). (*E*)-3-[4-((*E*)-2-Ethoxycarbonylvinyl)phenyl]-acrylic acid (1.5 g, 6.1 mmol) was then dissolved in CH_2Cl_2 (80 mL) and TEA (1.7 mL, 12 mmol). EDC (2.33 g, 12.2 mmol) and HOBt (1.65 g, 12.2 mmol) were added to the resulting mixture. After 15 min, NH_2OTHP (856 mg, 7.32 mmol) was added and the resulting mixture was stirred at room temperature for 5 h. The solution was partitioned between 5% $NaHCO_3$ and Et_2O. The organic phase was dried over Na_2SO_4, evaporated in vacuo, and purified by column chromatography (petroleum ether/EtOAc 1:1) to give the tetrahydropyran-2-yl-protected acrylamide **13** (1.94 g, 92%). ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 11.24 (bs, 1H), 7.77 (d, J = 8.22 Hz, 2H), 7.66 (d, J = 15.85 Hz, 1H), 7.57–7.65 (m, 2H), 7.50 (d, J = 16.14 Hz, 1H), 6.68 (d, J = 16.14 Hz, 1H), 6.58 (d, J = 16.43 Hz, 1H), 4.92 (bs, 1H), 4.20 (q, J = 7.04 Hz, 2H), 3.96 (bs, 1H), 3.43–3.66 (m, 1H), 1.38–1.96 (m, 6H), 1.27 ppm (t, J = 7.04 Hz, 3H).

LiOH (1 M, 3 mL) was added to a stirred solution of **13** (520 mg, 1.5 mmol) in THF (10 mL) and the resulting mixture was stirred at room temperature overnight. Further 1 M LiOH (1.5 mL) was added, and, after stirring at room temperature overnight, the solution was partitioned between H_2O and EtOAc. The aqueous phase was

brought to acidic pH by adding citric acid (5% aqueous solution) at 0 °C and extracted twice with EtOAc. The organic layers were washed with brine, dried over Na_2SO_4, and evaporated in vacuo to give the acrylic acid **15** as a white powder (445 mg, 94%). ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 12.35 (bs, 1H), 11.23 (bs, 1H), 7.69–7.84 (m, 2H), 7.58–7.67 (m, 2H), 7.59 (d, J = 16.14 Hz, 1H), 7.51 (d, J = 15.85 Hz, 1H), 6.57 (d, J = 16.14 Hz, 2H), 4.92 (bs, 1H), 3.97 (bs, 1H), 3.43–3.71 (m, 1H), 1.31–1.95 ppm (m, 6H).

A mixture of **15** (200 mg, 0.63 mmol), EDC (240 mg, 1.26 mmol), HOBt (170 mg, 1.26 mmol), TEA (0.354 mL, 2.52 mmol), and 1-phenylpiperazine (192 μL, 1.26 mmol) in CH_2Cl_2 (6 mL) and DMF (1 mL) was stirred overnight at room temperature. The mixture was then partitioned between H_2O and CH_2Cl_2. The aqueous phase was extracted with CH_2Cl_2 and the collected organic layers were dried over Na_2SO_4 and evaporated to dryness. The crude mixture was purified by column chromatography (CH_2Cl_2/MeOH/NH_4OH 90:10:0.1). The resulting product was dissolved in CH_2Cl_2 (10 mL) and treated with HCl/Et_2O for 1 h. The precipitate was filtered off and triturated with CH_2Cl_2 and Et_2O to give the desired hydroxamic acid **17b** (166 mg, 63% from **15**) as its HCl salt. ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 7.78 (m, 2H), 7.60 (m, 2H), 7.54 (d, J = 15.26 Hz, 1H), 7.46 (d, J = 15.85 Hz, 1H), 7.37 (d, J = 15.26 Hz, 1H), 7.27–7.34 (m, 2H), 7.10–7.23 (m, 2H), 6.87–7.05 (m, 1H), 6.57 (d, J = 15.85 Hz, 1H), 3.85 (bs, 4H), 3.27 ppm (bs, 4H); ^{13}C NMR (75 MHz, [D$_6$]DMSO): δ = 164.97, 162.98, 141.74, 137.74, 136.56, 136.46, 129.98 (2C), 129.12 (2C), 128.28 (2C), 120.70, 119.35 (2C), 119.02, 52.03 (2C), 44.01, 40.58 ppm; HRMS-TOF (ESI): m/z $[M+H]^+$ calcd for $C_{22}H_{24}N_3O_3$: 378.1817, found: 378.1803; HPLC purity: 100%, t_R = 1.56 min (Method 2).

(*E*)-*N*-Hydroxy-3-{4-[(*E*)-3-(4-methylpiperazin-1-yl)-3-oxo-1-propen-1-yl]phenyl}acrylamide (17a): Compound **17a** (35 mg, yield 18%) was obtained as its HCl salt starting from the THP-protected hydroxamic acid **15** and 1-methylpiperazine following the synthetic procedure of the hydroxamic acid **17b**. ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 10.83 (bs, 1H), 7.77 (d, J = 8.22 Hz, 2H), 7.60 (d, J = 8.22 Hz, 2H), 7.55 (d, J = 15.55 Hz, 1H), 7.46 (d, J = 15.85 Hz, 1H), 7.32 (d, J = 15.55 Hz, 1H), 6.54 (d, J = 15.85 Hz, 1H), 4.53 (d, J = 13.50 Hz, 2H), 3.23–3.56 (m, 4H), 2.88–3.13 (m, 2H), 2.78 ppm (d, J = 3.81 Hz, 3H); LC–MS (ESI): m/z: 316 $[M+H]^+$; HPLC purity: 99%, t_R = 2.31 min (Method 1).

(*E*)-*N*-Hydroxy-3-{5-[(*E*)-3-(4-phenylpiperazin-1-yl)-3-oxo-1-propen-1-yl]pyridin-2-yl}acrylamide (18b): A solution of triethyl phosphonoacetate (373 mg, 1.66 mmol) and NaH (60% oil dispersion, 71.7 mg, 1.79 mmol) in dry THF (5 mL) was added dropwise to a stirred solution of *tert*-butyl-(*E*)-3-(5-formylpyridin-2-yl)acrylate[28] (**10**, 323 mg, 1.38 mmol) in dry THF (5 mL) under N_2 atmosphere. The resulting mixture was stirred at room temperature for 4 h and was then partitioned between H_2O and Et_2O. The aqueous layer was washed with Et_2O, the collected organic phases were dried over Na_2SO_4, and evaporated in vacuo to give the diester **12** (420 mg, quant). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.77 (d, J = 2.05 Hz, 1H), 7.86 (dd, J = 8.22, 2.35 Hz, 1H), 7.68 (d, J = 16.14 Hz, 1H), 7.60 (d, J = 15.55 Hz, 1H), 7.45 (d, J = 7.92 Hz, 1H), 6.89 (d, J = 15.55 Hz, 1H), 6.54 (d, J = 16.14 Hz, 1H), 4.31 (q, J = 7.14 Hz, 2H), 1.56 (s, 9H), 1.37 ppm (t, J = 7.19 Hz, 3H).

A mixture of **12** (300 mg, 0.99 mmol) and TFA (4 mL) in CH_2Cl_2 (10 mL) was stirred at room temperature for 3 h. The solvent was removed to dryness and the residue (380 mg) was dissolved in CH_2Cl_2 (20 mL) and TEA (1.17 mL, 8.39 mmol). EDC (401 mg, 2.09 mmol), HOBt (283 mg, 2.10 mmol), and NH_2OTHP (184 mg, 1.57 mmol) were added and the mixture was stirred at room temperature for 4 h. Further NH_2OTHP (40 mg, 0.34 mmol) was added and the mixture was stirred overnight. The solvent was then evaporated and the residue was partitioned between H_2O and EtOAc. The organic layer was dried over Na_2SO_4 and evaporated to dryness. The crude mixture was purified by column chromatography (petroleum ether/EtOAc 4:6) to give the THP-protected hydroxamic acid **14** as a yellow powder (237 mg, 69%). ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 11.39 (bs, 1H), 8.92 (d, J = 2.05 Hz, 1H), 8.22 (dd, J = 8.07, 2.20 Hz, 1H), 7.27–7.88 (m, 3H), 7.02 (d, J = 15.26 Hz, 1H), 6.82 (d, J = 16.14 Hz, 1H), 4.94 (bs, 1H), 4.21 (q, J = 7.24 Hz, 2H), 3.84–4.09 (m, 1H), 3.46–3.63 (m, 1H), 1.41–1.86 (m, 6H), 1.27 ppm (t, J = 7.04 Hz, 3H).

LiOH (1 M, 1.68 mL) was added to a stirred solution of **14** (292 mg, 0.843 mmol) in THF (8 mL) and the resulting mixture was stirred at room temperature. Further 1 M LiOH (1.26 mL) was added over 20 h. The solution was then brought to pH 6 by slow addition of a 4 M HCl solution and then brought to basic pH value by adding NH_4OH. The solvent was concentrated and the residue was freeze-dried. The crude acrylic acid **16** was dissolved in CH_2Cl_2 (8 mL), DMF (8 mL), and TEA (0.234 mL, 1.68 mmol). EDC (322 mg, 1.68 mmol), HOBt (252 mg, 1.68 mmol), and 1-phenylpiperazine (164 mg, 1.01 mmol) were added and the resulting slurry was stirred at room temperature overnight. Further 1-phenylpiperazine (136 mg, 0.838 mmol) was added and the mixture was heated until dissolution of the slurry. After stirring for additional 6 h at room temperature the solution was partitioned between H_2O and EtOAc. The resulting precipitate was filtered off and washed with EtOAc and CH_2Cl_2. The obtained solid was suspended in CH_2Cl_2 and treated with HCl/Et_2O for 4 h. The resulting precipitate was filtered off and washed with CH_2Cl_2 to give the desired hydroxamic acid **18b** as its HCl salt (102 mg, 30% from **14**). ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 8.96 (d, J = 1.76 Hz, 1H), 8.34 (dd, J = 8.07, 1.91 Hz, 1H), 7.73 (d, J = 8.22 Hz, 1H), 7.44–7.67 (m, 3H), 7.27–7.41 (m, 2H), 7.10–7.25 (m, 2H), 7.01 (d, J = 15.55 Hz, 1H), 6.92–7.02 (m, 1H), 3.67–4.15 (m, 4H), 3.30 ppm (bs, 4H); ^{13}C NMR (75 MHz, [D$_6$]DMSO): δ = 164.46, 161.97, 154.02, 152.54, 148.41, 137.84, 137.70, 135.28, 132.10, 129.90 (2C), 125.70, 124.71, 121.66, 118.75 (2C), 51.45 (2C), 44.47, 40.88 ppm; HRMS-TOF (ESI): m/z $[M+H]^+$ calcd for $C_{21}H_{23}N_4O_3$: 379.1770, found: 379.1759; HPLC purity: 99%, t_R = 1.54 min (Method 5).

(*E*)-*N*-Hydroxy-3-{5-[(*E*)-3-(4-methylpiperazin-1-yl)-3-oxo-1-propen-1-yl]pyridin-2-yl}acrylamide (18a): Compound **18a** (52 mg, yield 19%) was obtained as its HCl salt by starting from the THP-protected hydroxamic acid **16** and 1-methylpiperazine following the synthetic procedure of the hydroxamic acid **18b**. ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 10.86 (bs, 1H), 8.92 (d, J = 1.76 Hz, 1H), 8.25 (dd, J = 8.22, 2.35 Hz, 1H), 7.67 (d, J = 7.92 Hz, 1H), 7.60 (d, J = 15.55 Hz, 1H), 7.51 (d, J = 15.26 Hz, 1H), 7.46 (d, J = 15.26 Hz, 1H), 6.99 (d, J = 15.26 Hz, 1H), 4.40–4.64 (m, 2H), 3.34–3.61 (m, 3H), 2.93–3.28 (m, 3H), 2.79 ppm (d, J = 4.40 Hz, 3H); LC–MS (ESI): m/z: 317 $[M+H]^+$; HPLC purity: 95%, t_R = 0.55 min (Method 2).

(*E*)-*N*-Hydroxy-3-{3-[(*E*)-3-(4-phenylpiperazin-1-yl)-3-oxo-1-propen-1-yl]phenyl}acrylamide (24b): A mixture of 3-bromobenzaldehyde (**19**, 500 mg, 2.70 mmol), TEA (1.62 mL, 11.6 mmol), ethyl acrylate (270 mg, 2.70 mmol), $NaHCO_3$ (454 mg, 5.4 mmol), and PPh_3 (35 mg, 0.13 mmol) in DMF (13 mL) was degassed with N_2 and then $Pd(OAc)_2$ (12 mg, 0.054 mmol) was added. The resulting slurry was heated at 100 °C under N_2 for 6 h and was then partitioned between H_2O and Et_2O. The organic phase was washed with H_2O, dried over Na_2SO_4, and evaporated to dryness. The crude reaction mixture was purified by column chromatography (petro-

leum ether/EtOAc 95:5) to give the ethyl acrylate **20** (206 mg, 37%). ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 10.04 (s, 1H), 8.26 (t, J = 1.61 Hz, 1H), 8.06 (dt, J = 7.70, 1.43 Hz, 1H), 7.94 (dt, J = 7.63, 1.32 Hz, 1H), 7.75 (d, J = 16.14 Hz, 1H), 7.65 (t, J = 7.63 Hz, 1H), 6.76 (d, J = 16.14 Hz, 1H), 4.22 (q, J = 7.04 Hz, 2H), 1.28 ppm (t, J = 7.04 Hz, 3H).

A solution of **20** (206 mg, 1.01 mmol) in dry THF (5 mL) was added dropwise under N_2 atmosphere to a stirred mixture of *tert*-butyldiethylphosphonoacetate (277 mg, 1.10 mmol) and NaH (60% oil dispersion, 52 mg, 1.3 mmol) in dry THF (10 mL), cooled to 0 °C. The resulting solution was stirred at room temperature for 45 min, then quenched with H_2O, and the mixture was extracted with EtOAc. The organic phase was dried, the solvent was removed in vacuo, and the crude mixture was purified by column chromatography (petroleum ether/EtOAc 95:5) to give the diester **21** (260 mg, 86%). ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 8.13 (s, 1H), 7.69–7.77 (m, 2H), 7.66 (d, J = 16.14 Hz, 1H), 7.56 (d, J = 16.14 Hz, 1H), 7.45 (t, J = 7.63 Hz, 1H), 6.78 (d, J = 15.85 Hz, 1H), 6.67 (d, J = 15.85 Hz, 1H), 4.21 (q, J = 7.04 Hz, 2H), 1.50 (s, 9H), 1.27 ppm (t, J = 7.19 Hz, 3H).

A mixture of the *tert*-butyl ester **21** (2.72 g, 9.00 mmol) and TFA (13.9 mL) in CH_2Cl_2 (28 mL) was stirred at room temperature for 1 h. The solvent was removed in vacuo to give (*E*)-3-[3-((*E*)-2-ethoxycarbonylvinyl)phenyl]acrylic acid (2.13 g, 96%). ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 12.41 (bs, 1H), 8.11 (s, 1H), 7.67–7.78 (m, 2H), 7.67 (d, J = 16.14 Hz, 1H), 7.60 (d, J = 16.14 Hz, 1H), 7.46 (t, J = 7.63 Hz, 1H), 6.77 (d, J = 16.14 Hz, 1H), 6.67 (d, J = 16.14 Hz, 1H), 4.21 (q, J = 7.04 Hz, 2H), 1.27 ppm (t, J = 7.04 Hz, 3H). The acrylic acid (2.13 g, 8.66 mmol) was then dissolved in a mixture of CH_2Cl_2 (80 mL) and TEA (2.4 mL, 17 mmol). EDC (3.31 g, 17.3 mmol) and HOBt (2.34 g, 17.3 mmol) were then added to the resulting solution. After 15 min NH_2OTHP (2.03 g, 17.3 mmol) was added and the resulting mixture was stirred at room temperature overnight. The solvent was evaporated and the residue was partitioned between 5% $NaHCO_3$ and Et_2O. The organic phase was dried over Na_2SO_4 and evaporated in vacuo. The crude mixture was purified by column chromatography (petroleum ether/EtOAc 1:1) to give the THP-protected hydroxamic acid **22** (2.62 g, 87%). ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 11.22 (bs, 1H), 7.95 (s, 1H), 7.66 (d, J = 15.85 Hz, 1H), 7.29–7.82 (m, 4H), 6.70 (d, J = 15.85 Hz, 1H), 6.60 (d, J = 15.85 Hz, 1H), 4.93 (bs, 1H), 4.20 (q, J = 7.24 Hz, 2H), 3.98 (d, J = 6.75 Hz, 1H), 3.54 (d, J = 11.44 Hz, 1H), 1.35–1.89 (m, 6H), 1.27 ppm (t, J = 7.04 Hz, 3H).

LiOH (1 M, 15.2 mL) was added to a stirred solution of **22** (2.62 g, 7.59 mmol) in THF (50 mL) and the mixture was stirred at room temperature overnight. Further 1 M LiOH (10 mL) was added and the reaction was carried out overnight. The solution was then partitioned between H_2O and EtOAc. The aqueous phase was brought to acidic pH by adding citric acid (5% aqueous solution) at 0 °C and was extracted twice with EtOAc. The organic layer was washed with brine, dried over Na_2SO_4, and evaporated in vacuo to give the acrylic acid **23** as a white powder (2.01 g, 83%). ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 11.22 (bs, 1H), 7.90 (s, 1H), 7.65–7.76 (m, 1H), 7.60 (d, J = 15.85 Hz, 1H), 7.32–7.65 (m, 3H), 6.60 (d, J = 16.14 Hz, 2H), 4.92 (bs, 1H), 3.80–4.15 (m, 1H), 3.46–3.69 (m, 1H), 1.31–1.95 ppm (m, 6H). The THP-protected hydroxamic acid **23** (250 mg, 0.79 mmol) was dissolved in CH_2Cl_2 (10 mL) and TEA (0.110 mL, 0.789 mmol). EDC (226 mg, 1.18 mmol), HOBt (159 mg, 1.18 mmol), and 1-phenylpiperazine (153 mg, 0.947 mmol) were added and the mixture was stirred overnight at room temperature. The resulting solution was partitioned between brine and CH_2Cl_2 and the aqueous phase was extracted with CH_2Cl_2. The collected organic layers were dried over Na_2SO_4 and evaporated to dryness. The crude product was purified by column chromatography (CH_2Cl_2/MeOH/NH_4OH from 99:1:0.1 to 98:2:0.2), and the resulting product was dissolved in CH_2Cl_2 (5 mL) and treated with HCl/Et_2O for 2 h. The precipitate was filtered off and washed with CH_2Cl_2 to give the desired hydroxamic acid **24b** as its HCl salt (238 mg, 73%). ^{1}H NMR (300 MHz, [D$_6$]DMSO, 353 K): δ = 7.87 (t, J = 1.61 Hz, 1H), 7.62–7.70 (m, 1H), 7.39–7.60 (m, 4H), 7.26–7.36 (m, 2H), 7.27 (t, J = 9.10 Hz, 1H), 7.06–7.18 (m, 2H), 6.86–6.98 (m, 1H), 6.64 (d, J = 15.85 Hz, 1H), 3.75–3.97 (m, 4H), 3.19–3.36 ppm (m, 4H); ^{13}C NMR (75 MHz, [D$_6$]DMSO): δ = 164.94, 163.02, 142.01, 138.15, 136.17, 135.98, 130.08 (s, 2C), 129.80, 129.55, 128.45, 127.91, 120.55, 119.76 (2C), 119.03, 52.39 (2C), 43.92, 40.85 ppm; HRMS-TOF (ESI): m/z $[M+H]^+$ calcd for $C_{22}H_{24}N_3O_3$: 378.1817, found: 378.1775; HPLC purity: 96%, t_R = 1.60 min (Method 4).

(*E*)-*N*-Hydroxy-3-{3-[(*E*)-3-(4-methylpiperazin-1-yl)-3-oxo-1-propen-1-yl]phenyl}acrylamide (24a): Compound **24a** (55 mg, yield 65%) was obtained as its HCl salt starting from THP protected hydroxamic acid **23** and 1-methylpiperazine following the synthetic procedure of the hydroxamic acid **24b**. ^{1}H NMR (300 MHz, [D$_6$]DMSO + TFA): δ = 10.30 (bs, 1H), 7.94 (s, 1H), 7.72 (d, J = 7.63 Hz, 1H), 7.41–7.63 (m, 4H), 7.34 (d, J = 15.26 Hz, 1H), 6.54 (d, J = 15.85 Hz, 1H), 4.55 (d, J = 13.20 Hz, 2H), 3.48 (d, J = 10.27 Hz, 3H), 2.94–3.22 (m, 3H), 2.82 ppm (s, 3H); LC–MS (ESI): m/z: 316 $[M+H]^+$; HPLC purity: 97%, t_R = 0.95 min (Method 3).

(±)-(*E*)-*N*-Hydroxy-3-{6-[(*E*)-3-(3-phenylpiperidin-1-yl)-3-oxo-1-propen-1-yl]pyridin-2-yl}acrylamide hydrochloride (29e): A solution of triethyl phosphonoacetate (3.34 g, 14.9 mmol) in dry THF (10 mL) was added dropwise under N_2 atmosphere to a stirred suspension of NaH (60% oil dispersion, 705 mg, 17.6 mmol) in dry THF (5 mL). The resulting mixture was stirred at room temperature for 15 min and then added dropwise, using a syringe equipped with a filtering septum, to a stirred solution of *tert*-butyl-(*E*)-3-(6-formylpyridin-2-yl)acrylate[28] (**25**, 3.16 g, 13.6 mmol) in dry THF (40 mL), keeping the temperature < 25 °C. The mixture was stirred at room temperature for 1.5 h, then diluted with EtOAc and washed with a saturated NH_4Cl solution. The aqueous phase was washed twice with EtOAc, the collected organic layers were dried over Na_2SO_4 and then evaporated to dryness. The crude product was purified by column chromatography (petroleum ether/EtOAc from 95:5 to 9:1) to give the diester **26** (3.92 g, 95%). ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 7.93 (t, J = 7.63 Hz, 1H), 7.76 (d, J = 7.63 Hz, 1H), 7.75 (d, J = 7.63 Hz, 1H), 7.67 (d, J = 15.55 Hz, 1H), 7.58 (d, J = 15.55 Hz, 1H), 6.98 (d, J = 15.85 Hz, 1H), 6.89 (d, J = 15.85 Hz, 1H), 4.23 (q, J = 7.04 Hz, 2H), 1.50 (s, 9H), 1.28 ppm (t, J = 7.04 Hz, 3H). A mixture of **26** (3.92 g, 12.9 mmol) and TFA (3.99 mL) in CH_2Cl_2 (50 mL) was stirred at room temperature for 24 h. Further TFA (2 mL) was added and the reaction mixture was stirred for additional 5 h at 30 °C. Then the solvent was removed in vacuo, the residue was triturated with Et_2O and decanted to give (*E*)-3-[6-((*E*)-2-ethoxycarbonylvinyl)pyridin-2-yl]acrylic acid as its trifluoroacetate salt (4.6 g, 98%). ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 12.58 (bs, 1H), 7.93 (t, J = 7.63 Hz, 1H), 7.76 (d, J = 7.04 Hz, 1H), 7.74 (d, J = 7.63 Hz, 1H), 7.68 (d, J = 15.85 Hz, 1H), 7.61 (d, J = 15.85 Hz, 1H), 6.98 (d, J = 15.55 Hz, 1H), 6.92 (d, J = 15.55 Hz, 1H), 4.23 (q, J = 7.24 Hz, 2H), 1.28 ppm (t, J = 7.19 Hz, 3H).

The acrylic acid (8.49 g, 23.5 mmol) was then dissolved in CH_2Cl_2 (150 mL) and TEA (3.27 mL, 23.5 mmol). EDC (4.49 g, 23.5 mmol), HOBt (3.18 g, 23.5 mmol), and NH_2OTHP (2.75 g, 23.5 mmol) were added and the resulting mixture was stirred at room temperature for 5 h. Further EDC (1.35 g, 7.07 mmol), HOBt (952 mg, 7.05 mmol), and NH_2OTHP (819 mg, 7.00 mmol) were added. The solution was stirred at room temperature overnight and was then

washed with H_2O and brine. The organic phase was dried over Na_2SO_4, evaporated to dryness, and the crude product was purified by column chromatography (petroleum ether/EtOAc from 65:35 to 45:55) to give the tetrahydropyran-2-yl-protected acrylamide **27** as a white powder (6.58 g, 81%). ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 11.39 (bs, 1H), 7.91 (t, J = 7.63 Hz, 1H), 7.71 (d, J = 7.34 Hz, 1H), 7.69 (d, J = 15.85 Hz, 1H), 7.61 (d, J = 7.63 Hz, 1H), 7.52 (d, J = 15.26 Hz, 1H), 7.10 (d, J = 15.55 Hz, 1H), 7.00 (d, J = 15.55 Hz, 1H), 4.94 (bs, 1H), 4.23 (q, J = 7.04 Hz, 2H), 3.80–4.04 (m, 1H), 3.41–3.68 (m, 1H), 1.42–1.87 (m, 6H), 1.28 ppm (t, J = 7.04 Hz, 3H).

NaOH (4 N, 11.38 mL) was added dropwise to a stirred solution of **27** (6.58 g, 19.0 mmol) in THF (200 mL). The mixture was stirred at room temperature for 2 h and was then brought to pH 5 with citric acid (20% aqueous solution). The solution was concentrated in vacuo, and the resulting slurry was acidified to pH 4 with citric acid and extracted with EtOAc and CH_2Cl_2 several times. The collected organic layers were dried over Na_2SO_4 and evaporated to dryness. The crude mixture was triturated in EtOAc, THF, and petroleum ether to give the acrylic acid **28** as a white powder (5.7 g, 95%). ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 12.30 (bs, 1H), 11.41 (bs, 1H), 7.90 (t, J = 7.63 Hz, 1H), 7.67 (d, J = 7.34 Hz, 1H), 7.60 (d, J = 7.63 Hz, 1H), 7.61 (d, J = 15.55 Hz, 1H), 7.54 (d, J = 15.26 Hz, 1H), 7.09 (d, J = 15.55 Hz, 1H), 6.93 (d, J = 15.55 Hz, 1H), 4.95 (bs, 1H), 3.84–4.11 (m, 1H), 3.36–3.74 (m, 1H), 1.28–2.01 ppm (m, 6H).

3-Phenylpiperidine (42.5 mg, 0.264 mmol) was added to a stirred solution of **28** (70 mg, 0.22 mmol), TEA (0.061 mL, 0.44 mmol), EDC (63 mg, 0.33 mmol), and HOBt (45 mg, 0.33 mmol) in CH_2Cl_2 (3 mL). The mixture was stirred at room temperature for 8 h and then washed with 1 M K_2CO_3. The layers were separated by a phase separator cartridge and the organic layer was shaken overnight in the presence of PS-isocyanate (240 mg, loading: 1.58 mmol g^{-1}). The resin was filtered off and the solvent was evaporated in vacuo. The crude product was purified using a SiO_2 cartridge (CH_2Cl_2/MeOH/NH_4OH 98:2:0.2) and the resulting product was dissolved in CH_2Cl_2 and treated with HCl/Et_2O for 2 h. The precipitate was filtered off to give the title compound as its HCl salt (44.4 mg, 53%). ^{1}H NMR (300 MHz, [D$_6$]DMSO, 353 K + TFA): δ = 7.86 (t, J = 7.63 Hz, 1H), 7.70 (d, J = 7.04 Hz, 1H), 7.55 (d, J = 15.85 Hz, 1H), 7.48–7.54 (m, 1H), 7.48 (d, J = 15.55 Hz, 1H), 7.46 (d, J = 15.26 Hz, 1H), 7.27–7.38 (m, 4H), 7.16–7.28 (m, 1H), 7.01 (d, J = 15.85 Hz, 1H), 4.12–4.45 (m, 2H), 3.04 (bs, 1H), 2.62–2.82 (m, 2H), 1.92–2.13 (m, 1H), 1.69–1.92 (m, 2H), 1.40–1.69 ppm (m, 1H); ^{13}C NMR (75 MHz, [D$_6$]DMSO): δ = 164.24, 162.13, 152.96, 143.95, 139.99, 128.93 (4C), 127.68, 127.46, 127.03, 124.50, 124.30, 52.07, 48.60, 46.24, 43.69, 42.56, 31.98, 26.76, 25.43 ppm; HRMS-TOF (ESI): m/z $[M+H]^+$ calcd for $C_{22}H_{24}N_3O_3$: 378.1817, found: 378.1850; HPLC purity: 98%, t_R = 1.83 min (Method 4).

(*E*)-*N*-Hydroxy-3-{6-[(*E*)-3-(4-methylpiperazin-1-yl)-3-oxo-1-propen-1-yl]pyridin-2-yl}acrylamide (29a): Compound **29a** (55 mg, yield 78%) was prepared by starting from the THP-protected hydroxamic acid **28** and 1-methylpiperazine following the synthetic procedure of the hydroxamic acid **29e**. ^{1}H NMR (300 MHz, [D$_6$]DMSO + TFA): δ = 10.68 (bs, 1H), 7.91 (t, J = 7.78 Hz, 1H), 7.77 (d, J = 7.34 Hz, 1H), 7.60 (d, J = 15.26 Hz, 1H), 7.57–7.61 (m, 1H), 7.54 (d, J = 15.26 Hz, 1H), 7.50 (d, J = 15.55 Hz, 1H), 7.03 (d, J = 15.55 Hz, 1H), 4.32–4.69 (m, 2H), 3.38–3.72 (m, 3H), 2.94–3.27 (m, 3H), 2.81 ppm (s, 3H); LC–MS (ESI): m/z: 317 $[M+H]^+$; HPLC purity: 98%, t_R = 0.81 min (Method 6).

(*E*)-*N*-Hydroxy-3-{6-[(*E*)-3-(4-phenylpiperazin-1-yl)-3-oxo-1-propen-1-yl]pyridin-2-yl}acrylamide (29b): Compound **29b** (yield 50%) was prepared by starting from the THP-protected hydroxamic acid **28** and 1-phenylpiperazine following the synthetic procedure of the hydroxamic acid **29e**. ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 7.93 (t, J = 7.63 Hz, 1H), 7.80 (d, J = 7.34 Hz, 1H), 7.66 (d, J = 15.26 Hz, 1H), 7.60 (d, J = 8.22 Hz, 1H), 7.55 (d, J = 15.55 Hz, 1H), 7.51 (d, J = 15.26 Hz, 1H), 7.32 (t, J = 8.07 Hz, 2H), 7.18 (d, J = 7.92 Hz, 2H), 7.04 (d, J = 15.26 Hz, 1H), 6.93–7.01 (m, 1H), 3.67–4.06 (m, 4H), 3.17–3.45 ppm (m, 4H); ^{13}C NMR (75 MHz, [D$_6$]DMSO): δ = 164.49, 162.31, 153.23, 153.04, 147.35, 140.63, 139.29, 136.71, 129.97 (2C), 124.91, 124.60 (2 C) 124.26, 123.20, 119.00 (2C), 51.51 (2C), 44.43, 40.85 ppm; HRMS-TOF (ESI): m/z $[M+H]^+$ calcd for $C_{21}H_{23}N_4O_3$: 379.1770, found: 379.1771; HPLC purity: 97%, t_R = 1.81 min (Method 4).

(±)-(*E*)-*N*-Hydroxy-3-{6-[(*E*)-3-(3-phenylpyrrolidin-1-yl)-3-oxo-1-propen-1-yl]pyridin-2-yl}acrylamide hydrochloride (29c): Compound **29c** (yield 28%) was prepared by starting from the THP-protected hydroxamic acid **28** and 3-phenylpyrrolidine according to the procedure described for the synthesis of **29e**. ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 7.90 (t, J = 7.92 Hz, 1H), 7.76 (d, J = 7.19 Hz, 1H), 7.13–7.65 (m, 9H), 7.00 (d, J = 15.55 Hz, 1H), 3.88–4.08 (m, 1H), 3.07–4.50 (m, 4H), 2.20–2.47 (m, 1H), 1.80–2.20 ppm (m, 1H); LC–MS (ESI): m/z: 364 $[M+H]^+$; HPLC purity: 95%, t_R = 1.64 min (Method 4).

(±)-(*E*)-*N*-Hydroxy-3-{6-[(*E*)-3-(2-phenylpiperidin-1-yl)-3-oxo-1-propen-1-yl]pyridin-2-yl}acrylamide hydrochloride (29d): Compound **29d** (yield 36%) was prepared by starting from the THP-protected hydroxamic acid **28** and 2-phenylpiperidine according to the synthetic procedure of the hydroxamic acid **29e**. ^{1}H NMR (300 MHz, [D$_6$]DMSO, 353 K): δ = 7.84 (t, J = 7.63 Hz, 1H), 7.64 (d, J = 7.63 Hz, 1H), 7.56 (d, J = 15.55 Hz, 1H), 7.48–7.56 (m, 1H), 7.49 (d, J = 15.55 Hz, 1H), 7.47 (d, J = 15.55 Hz, 1H), 7.33–7.43 (m, 2H), 7.20–7.34 (m, 3H), 7.00 (d, J = 15.55 Hz, 1H), 5.44–5.95 (m, 1H), 3.90–4.48 (m, 1H), 2.79–3.13 (m, 1H), 2.29–2.47 (m, 1H), 1.81–2.08 (m, 1H), 1.35–1.81 ppm (m, 4H); LC–MS (ESI): m/z: 378 $[M+H]^+$; HPLC purity: 95%, t_R = 1.79 min (Method 4).

(*E*)-*N*-Hydroxy-3-{6-[(*E*)-3-(4-phenylpiperidin-1-yl)-3-oxo-1-propen-1-yl]pyridin-2-yl}acrylamide hydrochloride (29f): Compound **29f** (yield 49%) was prepared by starting from the THP-protected hydroxamic acid **28** and 4-phenylpiperidine according to the methodology described for the hydroxamic acid **29e**. ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 7.90 (t, J = 7.78 Hz, 1H), 7.77 (d, J = 7.04 Hz, 1H), 7.63 (d, J = 15.55 Hz, 1H), 7.57 (d, J = 7.34 Hz, 1H), 7.51 (d, J = 15.26 Hz, 1H), 7.50 (d, J = 15.55 Hz, 1H), 7.14–7.38 (m, 5H), 7.01 (d, J = 15.26 Hz, 1H), 4.54–4.83 (m, 1H), 4.21–4.46 (m, 1H), 3.05–3.40 (m, 1H), 2.67–2.96 (m, 2H), 1.77–2.05 (m, 2H), 1.41–1.74 ppm (m, 2H); LC–MS (ESI): m/z: 378 $[M+H]^+$; HPLC purity: 99%, t_R = 1.81 min (Method 4).

(±)-(*E*)-*N*-Hydroxy-3-{6-[(*E*)-3-(3-phenylpiperazin-1-yl)-3-oxo-1-propen-1-yl]pyridin-2-yl}acrylamide hydrochloride (29g): Compound **29g** (yield 28%) was prepared by starting from the THP-protected hydroxamic acid **28** according to the methodology described for the hydroxamic acid **29e**. ^{1}H NMR (300 MHz, [D$_6$]DMSO, 353 K + Na_2CO_3): δ = 10.76 (bs, 1H), 8.82 (bs, 1H), 7.86 (t, J = 7.78 Hz, 1H), 7.67–7.76 (m, 1H), 7.56–7.64 (m, 2H), 7.29–7.57 (m, 7H), 7.05 (d, 1H), 4.29–4.64 (m, 2H), 4.05 (dd, J = 10.86, 2.64 Hz, 1H), 3.39 (dd, J = 10.71, 4.55 Hz, 1H), 3.21–3.32 (m, 1H), 2.92–3.03 ppm (m, 2H); LC–MS (ESI): m/z: 379 $[M+H]^+$; HPLC purity: 98%, t_R = 1.01 min (Method 4).

(±)-(*E*)-*N*-Hydroxy-3-{6-[(*E*)-3-(4-methyl-3-phenylpiperazin-1-yl)-3-oxo-1-propen-1-yl]pyridin-2-yl}acrylamide hydrochloride (29h): Compound **29h** (yield 38%) was prepared by starting from the THP-protected hydroxamic acid **28** according to the synthetic

procedure of the hydroxamic acid **29e**. ^{1}H NMR (300 MHz, [D$_6$]DMSO, 353 K): δ = 7.87 (t, J = 7.63 Hz, 1H), 7.72 (d, J = 7.34 Hz, 1H), 7.67–7.83 (m, 2H), 7.38–7.62 (m, 7H), 7.04 (d, J = 15.85 Hz, 1H), 4.16–4.72 (m, 3H), 3.80–4.10 (m, 1H), 3.46–3.81 (m, 2H), 3.27 (td, J = 12.18, 3.81 Hz, 1H), 2.52 ppm (s, 3H); LC–MS (ESI): m/z: 393 [M-H]$^+$; HPLC purity: 97%, t_R = 1.48 min (Method 6).

(±)-(*E*)-*N*-Hydroxy-3-{6-[(*E*)-3-(4-phenylazepan-1-yl)-3-oxo-1-propen-1-yl]pyridin-2-yl}acrylamide hydrochloride (29i): Compound **29i** (yield 24%) was prepared by starting from the THP-protected hydroxamic acid **28** and 4-phenylazepane according to the methodology described for the hydroxamic acid **29e**. ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 7.82–8.00 (m, 1H), 7.77 (d, J = 7.19 Hz, 1H), 7.40–7.69 (m, 4H), 7.08–7.40 (m, 5H), 6.99 (d, J = 15.41 Hz, 1H), 3.25–4.12 (m, 4H), 2.59–2.82 (m, 1H), 1.90–2.10 (m, 2H), 1.62–1.88 ppm (m, 4H); LC–MS (ESI): m/z: 392 [M+H]$^+$; HPLC purity: 98%, t_R = 1.84 min (Method 4).

(+)-(*E*)-*N*-Hydroxy-3-{6-[(*E*)-3-((*R*)-3-phenylpiperidin-1-yl)-3-oxo-1-propen-1-yl]pyridin-2-yl}acrylamide hydrochloride (29j): Compound **29j** (yield 56%) was prepared by starting from the THP-protected hydroxamic acid **28** and (*R*)-3-phenylpiperidine according to the methodology described for the hydroxamic acid **29e**. $[\alpha]_D$ = +137.5 (c = 0.5 in MeOH); ^{1}H NMR (300 MHz, MeOD, 333 K): δ = 8.25 (t, J = 7.92 Hz, 1H), 8.01 (d, J = 7.63 Hz, 1H), 7.94 (d, J = 8.22 Hz, 1H), 7.52–7.82 (m, 3H), 7.18–7.39 (m, 5H), 7.07 (bs, 1H), 4.66 (bs, 1H), 4.30 (bs, 1H), 3.33–3.43 (m, 1H), 2.74–2.98 (m, 2H), 2.06–2.14 (m, 1H), 1.84–2.02 (m, 2H), 1.63–1.78 ppm (m, 1H); ^{13}C NMR (75 MHz, [D$_6$]DMSO): δ = 164.24, 162.13, 152.96, 143.95, 139.99, 128.93 (4C), 127.68, 127.46, 127.03, 124.50, 124.30, 52.07, 48.60, 46.24, 43.69, 42.56, 31.98, 26.76, 25.43 ppm; HRMS-TOF (ESI): m/z [M+H]$^+$ calcd for $C_{22}H_{24}N_3O_3$: 378.1817, found: 378.1794; HPLC purity: 99%, t_R = 1.78 min (Method 4).

(−)-(*E*)-*N*-Hydroxy-3-{6-[(*E*)-3-((*S*)-3-phenylpiperidin-1-yl)-3-oxo-1-propen-1-yl]pyridin-2-yl}acrylamide hydrochloride (29k): Compound **29k** (yield 29%) was prepared by starting from the THP-protected hydroxamic acid **28** and (*S*)-3-phenylpiperidine according to the methodology described for the hydroxamic acid **29e**. $[\alpha]_D$ = −112.9 (c = 0.5 in MeOH); ^{1}H NMR (300 MHz, [D$_6$]DMSO, 353 K): δ = 7.85 (t, J = 7.78 Hz, 1H), 7.68 (d, J = 7.04 Hz, 1H), 7.39–7.64 (m, 4H), 7.16–7.39 (m, 5H), 7.02 (d, J = 15.26 Hz, 1H), 3.63–4.83 (m, 1H), 2.85–3.32 (m, 2H), 2.56–2.85 (m, 2H), 1.94–2.11 (m, 1H), 1.68–1.94 (m, 2H), 1.39–1.70 ppm (m, 1H); ^{13}C NMR (75 MHz, [D$_6$]DMSO): δ = 164.24, 162.13, 152.96, 143.95, 139.99, 128.93 (4C), 127.68, 127.46, 127.03, 124.50, 124.30, 52.07, 48.60, 46.24, 43.69, 42.56, 31.98, 26.76, 25.43 ppm; HRMS-TOF (ESI): m/z [M+H]$^+$ calcd for $C_{22}H_{24}N_3O_3$: 378.1817, found: 378.1816; HPLC purity: 95%, t_R = 1.78 min (Method 4).

(±)-(*E*)-*N*-Hydroxy-3-(6-{(*E*)-3-[3-(2-fluorophenyl)piperidin-1-yl]-3-oxo-1-propen-1-yl}pyridin-2-yl)acrylamide hydrochloride (29l): Compound **29l** (yield 53%) was prepared by starting from the THP-protected hydroxamic acid **28** and 3-(2-fluorophenyl)piperidine according to the procedure described for the hydroxamic acid **29e**. ^{1}H NMR (300 MHz, [D$_6$]DMSO, 353 K): δ = 7.85 (t, J = 7.78 Hz, 1H), 7.66 (d, J = 7.63 Hz, 1H), 7.37–7.57 (m, 5H), 7.11–7.34 (m, 3H), 7.02 (d, J = 15.85 Hz, 1H), 4.16–4.49 (m, 2H), 2.95–3.20 (m, 3H), 1.79–2.03 (m, 3H), 1.54–1.69 ppm (m, 1H); ^{13}C NMR (75 MHz, [D$_6$]DMSO): δ = 164.34, 162.24, 162.15, 153.20, 152.71, 139.59 (2C), 136.39, 130.14, 128.94, 128.83, 128.77, 125.12, 124.23 (3C), 115.84, 47.29, 46.22, 35.76, 30.62, 26.71 ppm; HRMS-TOF (ESI): m/z [M+H]$^+$ calcd for $C_{22}H_{23}FN_3O_3$: 396.1724, found: 396.1750; HPLC purity: 99%, t_R = 1.82 min (Method 4).

(±)-(*E*)-*N*-Hydroxy-3-(6-{(*E*)-3-[3-(3-fluorophenyl)piperidin-1-yl]-3-oxo-1-propen-1-yl}pyridin-2-yl)acrylamide hydrochloride (29m): Compound **29m** (yield 64%) was prepared by starting from the THP-protected hydroxamic acid **28** and 3-(3-fluorophenyl)piperidine according to the procedure described for the hydroxamic acid **29e**. ^{1}H NMR (300 MHz, [D$_6$]DMSO, 353 K): δ = 7.85 (t, J = 7.78 Hz, 1H), 7.68 (dd, J = 7.92, 0.88 Hz, 1H), 7.29–7.59 (m, 5H), 7.10–7.19 (m, 2H), 6.97–7.07 (m, 2H), 4.23–4.55 (m, 2H), 2.98–3.17 (m, 2H), 2.67–2.90 (m, 1H), 1.93–2.14 (m, 1H), 1.71–1.90 (m, 2H), 1.52–1.66 ppm (m, 1H); LC–MS (ESI): m/z: 396 [M+H]$^+$; HPLC purity: 98%, t_R = 1.84 min (Method 4).

(±)-(*E*)-*N*-Hydroxy-3-(6-{(*E*)-3-[3-(4-fluorophenyl)piperidin-1-yl]-3-oxo-1-propen-1-yl}pyridin-2-yl)acrylamide hydrochloride (29n): Compound **29n** (yield 44%) was prepared by starting from the THP-protected hydroxamic acid **28** and 3-(4-fluorophenyl)piperidine according to the procedure described for the hydroxamic acid **29e**. ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 7.75–8.09 (m, 2H), 7.27–7.75 (m, 6H), 6.66–7.26 (m, 3H), 4.38–4.77 (m, 1H), 3.87–4.39 (m, 1H), 3.02–3.47 (m, 1H), 2.53–3.01 (m, 2H), 1.20–2.18 ppm (m, 4H); LC–MS (ESI): m/z: 396 [M+H]$^+$; HPLC purity: 96%, t_R = 1.82 min (Method 4).

(±)-(*E*)-*N*-Hydroxy-3-{6-[(*E*)-3-(3-(*o*-tolyl)piperidin-1-yl)-3-oxo-1-propen-1-yl]pyridin-2-yl}acrylamide hydrochloride (29o): Compound **29o** (yield 67%) was prepared by starting from the THP-protected hydroxamic acid **28** and 3-(*o*-tolyl)piperidine following the synthetic procedure of the hydroxamic acid **29e**. ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 6.56–8.27 (m, 11H), 4.41–4.74 (m, 1H), 3.96–4.41 (m, 1H), 3.01–3.36 (m, 1H), 2.56–3.03 (m, 2H), 2.32 (s, 3H), 1.69–2.06 (m, 3H), 1.34–1.69 ppm (m, 1H); LC–MS (ESI): m/z: 392 [M+H]$^+$; HPLC purity: 94%, t_R = 1.90 min (Method 4).

(±)-(*E*)-*N*-Hydroxy-3-{6-[(*E*)-3-(3-(*m*-tolyl)piperidin-1-yl)-3-oxo-1-propen-1-yl]pyridin-2-yl}acrylamide hydrochloride (29p): Compound **29p** (yield 40%) was prepared by starting from the THP-protected hydroxamic acid **28** and 3-(*m*-tolyl)piperidine following the synthetic procedure of the hydroxamic acid **29e**. ^{1}H NMR (300 MHz, [D$_6$]DMSO + Na_2CO_3): δ = 9.83 (bs, 1H), 7.27–7.93 (m, 6H), 7.22 (t, J = 7.34 Hz, 1H), 6.78–7.15 (m, 4H), 4.39–4.80 (m, 1H), 4.02–4.40 (m, 1H), 2.92–3.26 (m, 1H), 2.53–2.90 (m, 2H), 2.30 (s, 3H), 1.65–2.08 (m, 3H), 1.28–1.66 ppm (m, 1H); LC–MS (ESI): m/z: 392 [M+H]$^+$; HPLC purity: 98%, t_R = 1.94 min (Method 4).

(±)-(*E*)-*N*-Hydroxy-3-{6-[(*E*)-3-(3-(*p*-tolyl)piperidin-1-yl)-3-oxo-1-propen-1-yl]pyridin-2-yl}acrylamide hydrochloride (29q): Compound **29q** (yield 50%) was prepared by starting from the THP-protected hydroxamic acid **28** and 3-(*p*-tolyl)piperidine following the synthetic procedure of the hydroxamic acid **29e**. ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 7.71–8.15 (m, 2H), 7.34–7.74 (m, 4H), 6.71–7.34 (m, 5H), 4.35–4.79 (m, 1H), 3.88–4.38 (m, 1H), 3.01–3.42 (m, 1H), 2.55–2.99 (m, 2H), 2.28 (s, 3H), 1.24–2.10 ppm (m, 4H); LC–MS (ESI): m/z: 392 [M+H]$^+$; HPLC purity: 97%, t_R = 1.94 min (Method 4).

(±)-(*E*)-*N*-Hydroxy-3-(6-{(*E*)-3-[3-(2-methoxyphenyl)piperidin-1-yl]-3-oxo-1-propen-1-yl}pyridin-2-yl)acrylamide hydrochloride (29r): Compound **29r** (yield 53%) was prepared by starting from the THP-protected hydroxamic acid **28** and 3-(2-methoxyphenyl)piperidine according to the procedure described for the hydroxamic acid **29e**. ^{1}H NMR (300 MHz, [D$_6$]DMSO, 353 K): δ = 7.74–7.94 (m, 1H), 7.35–7.75 (m, 5H), 7.10–7.35 (m, 2H), 6.89–7.12 (m, 2H), 6.88 (d, J = 15.85 Hz, 1H), 3.99–4.84 (m, 2H), 3.54–3.99 (m, 3H), 2.71–3.38 (m, 3H), 1.72–2.23 (m, 3H), 1.30–1.72 ppm (m, 1H); LC–MS (ESI): m/z: 408 [M+H]$^+$; HPLC purity: 98%, t_R = 1.86 min (Method 4).

(±)-(*E*)-*N*-Hydroxy-3-(6-{(*E*)-3-[3-(3-methoxyphenyl)piperidin-1-yl]-3-oxo-1-propen-1-yl}pyridin-2-yl)acrylamide hydrochloride (29s): Compound **29s** (yield 47%) was prepared by starting from the THP-protected hydroxamic acid **28** and 3-(3-methoxyphenyl)piperidine according to the procedure described for the hydroxamic acid **29e**. ^{1}H NMR (300 MHz, MeOD, 333 K): δ = 8.38 (dd, J = 7.92 Hz, 1H), 7.99–8.23 (m, 2H), 7.59–7.86 (m, 3H), 7.25 (dd, J = 7.92 Hz, 1H), 7.10 (d, J = 16.14 Hz, 1H), 6.74–6.96 (m, 3H), 4.64 (bs, 1H), 4.29 (bs, 1H), 3.80 (s, 3H), 3.21–3.48 (m, 1H), 2.71–3.05 (m, 2H), 2.05–2.15 (m, 1H), 1.80–2.02 (m, 2H), 1.62–1.77 ppm (m, 1H); LC–MS (ESI): *m/z*: 408 $[M+H]^+$; HPLC purity: 98%, t_R = 1.78 min (Method 4).

(±)-(*E*)-*N*-Hydroxy-3-(6-{(*E*)-3-[3-(4-methoxyphenyl)piperidin-1-yl]-3-oxo-1-propen-1-yl}pyridin-2-yl)acrylamide hydrochloride (29t): Compound **29t** (yield 41%) was prepared by starting from the THP-protected hydroxamic acid **28** and 3-(4-methoxyphenyl)piperidine according to the procedure described for the hydroxamic acid **29e**. ^{1}H NMR (300 MHz, [D$_6$]DMSO, 353 K + TFA): δ = 7.85 (dd, J = 7.63 Hz, 1H), 7.60–7.73 (m, 1H), 7.36–7.60 (m, 4H), 7.22 (m, 2H), 7.02 (d, J = 15.26 Hz, 1H), 6.90 (m, 2H), 4.34 (bs, 2H), 3.75 (s, 3H), 3.00 (bs, 2H), 2.59–2.80 (m, 1H), 1.92–2.07 (m, 1H), 1.78–1.92 (m, 1H), 1.66–1.78 (m, 1H), 1.48–1.66 ppm (m, 1H); LC–MS (ESI): *m/z*: 408 $[M+H]^+$; HPLC purity: 99%, t_R = 1.76 min (Method 4).

(±)-(*E*)-*N*-Hydroxy-3-{6-[(*E*)-3-(3-naphthalen-1-ylpiperidin-1-yl)-3-oxo-1-propen-1-yl]pyridin-2-yl}acrylamide hydrochloride (29u): Compound **29u** (yield 46%) was prepared by starting from the THP-protected hydroxamic acid **28** and 3-naphthalen-1-ylpiperidine following the synthetic procedure of the hydroxamic acid **29e**. ^{1}H NMR (300 MHz, [D$_6$]DMSO, 353 K): δ = 8.04–8.41 (m, 1H), 7.28–8.03 (m, 12H), 6.78–7.23 (m, 1H), 4.05–4.94 (m, 2H), 3.25–3.77 (m, 1H), 2.79–3.39 (m, 2H), 1.52–2.27 ppm (m, 4H); LC–MS (ESI): *m/z*: 428 $[M+H]^+$; HPLC purity: 97%, t_R = 3.11 min (Method 6).

(±)-(*E*)-*N*-Hydroxy-3-{6-[(*E*)-3-(3-naphthalen-2-ylpiperidin-1-yl)-3-oxo-1-propen-1-yl]pyridin-2-yl}acrylamide hydrochloride (29v): Compound **29v** (yield 47%) was prepared by starting from the THP-protected hydroxamic acid **28** and 3-naphthalen-2-ylpiperidine following the synthetic procedure of the hydroxamic acid **29e**. ^{1}H NMR (300 MHz, [D$_6$]DMSO, 353 K): δ = 7.75–7.98 (m, 5H), 7.32–7.77 (m, 8H), 7.02 (d, J = 15.85 Hz, 1H), 4.02–4.79 (m, 2H), 2.99–3.46 (m, 2H), 2.76–3.00 (m, 1H), 2.03–2.23 (m, 1H), 1.79–2.04 (m, 2H), 1.44–1.82 ppm (m, 1H); LC–MS (ESI): *m/z*: 428 $[M+H]^+$; HPLC purity: 97%, t_R = 3.16 min (Method 6).

Biological assays

Pan-HDAC inhibition assay: Pan-HDAC inhibition assays were performed as previously described.[28]

HDAC1, 3, 4, 6, and 8 inhibition assays: Assays for inhibition of HDAC1, 3, 4, 6, and 8 were carried out by Amphora Discovery Corp. (Research Triangle Park, NC, USA) as previously described.[40,33] Purified HDACs were incubated with the test compounds and a carboxyfluorescein-labeled peptide (1 μM) as substrate for 17 h at 25 °C in a buffer consisting of 100 mM HEPES (pH 7.5), 1 mg mL^{-1} BSA, 0.01% Triton X-100, 1% DMSO, and 25 mM KCl. The reaction was stopped by the addition of 45 μL 0.08% sodium dodecyl sulfate in 100 mM HEPES (pH 7.5), and the substrate and product were then separated electrophoretically using a LabChip 3000 system (Caliper Life Sciences, Hopkinton, MA, USA) with blue laser excitation and green fluorescence detection. The fluorescence intensity was established using the Well Analyzer software on the Caliper system. All experiments were performed in duplicate. IC_{50} values were determined from 12-point concentration–response curves using a nonlinear regression analysis.

Cell growth assay: The antiproliferative effect of the HDAC inhibitors on cell proliferation was evaluated against K562 (chronic myeloid carcinoma), A549 (non-small-cell lung cancer) and HCT-116 (human colon cancer) cell lines using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. K562, A549, and HCT-116 cells were incubated for 72 h with various inhibitor concentrations. An equivalent of the CellTiter-Glo reagent was then added, the solution was mixed for 2 min in order to induce cell lysis, and the luminescence was recorded after a further 10 min. IC_{50} values were calculated using GraphPad software.

Metabolic stability in hepatic microsomes and hepatocytes: *Sample preparation:* By adapting the protocols described by Di et al.,[41] the compounds at 1 μM concentration were pre-incubated for 10 min at 37 °C in potassium phosphate buffer (pH 7.4) together with either 0.5 mg mL^{-1} mouse or human hepatic microsomes (Xenotech, Kansas City, MO, USA). The cofactor mixture comprising NADP, G6P and G6P-DH was added, and aliquots were taken after 0 and 30 min, quenched with CH_3CN, separated by centrifugation, and analyzed by LC–MS–MS.

Rat or human cryogenically preserved hepatocytes (Xenotech, Kansas City, MO, USA) were thawed and isolated according to the manufacturer's protocol using the XenoTech Hepatocyte Isolation Kit. The inhibitors were incubated at 2.5 μM in 0.5×10^6 cells μL^{-1} at 37 °C. Aliquots, taken at 0, 15, 30, 60, and 90 min, were quenched with ice-cold CH_3CN, centrifuged, and the supernatant was analyzed by LC–MS–MS.

Sample analysis: LC–MS–MS analyses were performed on an Acquity UPLC, coupled with a sample organizer and interfaced with a triple quadrupole Premiere XE (Waters, Milford, USA). Mobile phases consisted of a phase A [0.1% formic acid in a mixture of H_2O and CH_3CN (95:5 *v/v*)] and phase B [0.1% formic acid in a mixture of H_2O and CH_3CN (5:95 *v/v*)]. LC runs were carried out at 40 °C on Acquity BEH C_{18} columns (1.7 μm, 50 mm × 2.1 mm at a flow rate of 0.45 mL min^{-1}, or 1.7 μm, 50 mm × 1 mm at a flow rate of 0.2 mL min^{-1}). The columns were conditioned with 2% phase B for 0.2 min, then brought to 100% phase B within 0.01 min and maintained at these conditions for 1.3 min. LC–MS–MS analyses were carried out using a positive electrospray ionization (ESI(+)) interface in multiple reaction monitoring (MRM) mode with verapamil as internal standard. The percentage of the compound remaining after 30 min incubation in microsomes was calculated according to the following equation: $[(AUC_{30\ min})/(AUC_{0\ min})]\times100\%$.

For the clearance studies of **24b**, **29b**, and **29e** in rat and human hepatocytes, MRM transitions of potentially formed amide and acrylic acid metabolites were also monitored. The rates of intrinsic clearance (CL_i) of the compounds were calculated according to the following equation: CL_i [μL min^{-1} (10^6 cells)$^{-1}$] = $k\times V$, for which V is the incubation volume per 10^6 cells and k (min^{-1}) is the rate constant derived from the exponential decay.

Histone acetylation assay: The histone acetylation assay was performed as previously described.[28]

In vivo drug pharmacokinetic studies: PK experiments were performed using four-week-old male nude CD-1 mice (Charles River Laboratories, Calco, Italy). Animals were quarantined for about one week prior to the study. They were housed under standard conditions and had free access to water and standard laboratory rodent

diet. Care and husbandry of animals were in conformity with the institutional guidelines, in compliance with Italian Law (D. L.vo 116/92).

The compounds were dissolved in a mixture of 3% DMSO and 10% Encapsin in H_2O at a concentration of 1 mg mL^{-1} for the i.v. (rapid bolus) administration or in H_2O (**29b**) or H_2O containing 5% DMSO and 9.5% Encapsin (**24b**, **29e**) for the oral (gavage) dose at a concentration of 3 mg mL^{-1}. Each experimental group contained 27–30 animals. The compounds were administered to mice either by i.v. or oral route, and blood samples were collected after various time points after dosing. Plasma was separated immediately after blood sampling by centrifugation; plasma proteins were precipitated using Sirocco filtration plates or Oasis HLB elution plates according to the distributor's instructions, and the plasma samples were kept frozen (−80 °C) until submission to LC–MS–MS analysis. Sample analyses were performed on an Acquity UPLC using either Acquity BEH C_{18} columns (1.7 μm, 50 mm × 2.1 mm) or Acquity HSS T3 columns (1.8 μm, 50 mm × 2.1 mm), coupled with a sample organizer and interfaced to a triple quadrupole Premiere XE (Waters, Milford, USA). LC–MS–MS analyses were carried out using an ESI(+) interface in MRM mode.

PK parameters were calculated by a non-compartmental method using WinNolin 5.1 software (Pharsight, Mountain View, CA, USA). Absolute oral bioavailability (*F*) was calculated using the relationship: $F = [\text{dose}_{\text{i.v.}} \times \text{AUC}_{\text{oral } 0-\infty} / \text{dose}_{\text{oral}} \times \text{AUC}_{\text{i.v. } 0-\infty}] \times 100$.

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